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RESEARCH ARTICLE

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Variable viral loads and immune response in an invasive ant's native and introduced ranges

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Abstract

Aim: Pathogens can play an important role in biological invasions. Introduced populations may be particularly vulnerable to pathogens due to factors such as low genetic diversity and high population density. However, introduced populations that escape their natural pathogens may reallocate resources away from immunity and towards growth and reproduction. Interestingly, introduced ants have been suggested to have increased tolerance to new pathogens, contributing to their success as introduced species. In this study, we aimed to investigate whether introduced Argentine ant populations harbour different viral loads compared to native populations and if these differences were related to immunity-related gene expression.

Location: The study was conducted across the native range of Argentine ants in Argentina and four introduced regions in California, France, Australia and New Zealand.

Methods: We used RT-qPCR to quantify viral loads and gene expression in the ants. We analysed 15 different potentially pathogenic viruses across the Argentine ant's native and introduced ranges.

Results: We found that five viruses, LhuPcV1, LhuPiLV1, LhuCV1, Kashmir Bee virus and LHUV-1, presented high loads in Argentine ants compared to the other viruses we screened. We found a significant effect of range on viral infections: high viral loads were commonly found in ants from introduced populations, which also exhibited increased immune gene expression. We found highly significant correlations between viral loads and expression of immune and metabolic genes. However, these associations were not fully consistent across the studied regions, indicating the complexity of eco-immunological phenomena.

Main Conclusions: Our results suggest that introduced Argentine ant populations host different viral communities compared to native populations and that these differences are correlated with changes in immunity-related gene expression. The study highlights the complex role of pathogens in biological invasions and the importance

of considering eco-immunological factors when assessing the impact of introduced species.

KEYWORDS
Argentine ant (*Linepithema humile*), Eco-immunology, Invasive species, Quantitative PCR, Viruses

1 | INTRODUCTION

Pathogens have been proposed to play a key role in driving biological invasions (Dunn et al., 2012; Lee & Klasing, 2004; Prenter et al., 2004; White & Perkins, 2012). The ecological success of biological invaders is hypothesised to be facilitated by the purging of their natural pathogens present in their native range through bottleneck events that result in low initial host densities and founder effects (i.e. enemy release hypothesis; Keane & Crawley, 2002; Torchin et al., 2003). The evolution of increased competitive ability hypothesis extends the enemy release hypothesis to predict that under reduced pressure from pathogens, physiological resources could be re-allocated away from costly immune defences and enhance the invader's fitness (Blossey & Notzold, 1995; Colautti et al., 2004). Simultaneously, introduced species can also acquire existing pathogens in their new range, or facilitate the emergence of new pathogens (Stricker et al., 2016). Although pathogens may lack specialisation to infect a novel host or take time to efficiently do so (Woolhouse et al., 2002), some viruses in particular are known to have the potential to jump between species and exhibit a wide host range (Dobelmann et al., 2020; Levitt et al., 2013). In some cases, invasive species, including ants, might be particularly susceptible to pathogen epidemics and suffer population collapse in their introduced range (Lester & Gruber, 2016; Simberloff & Gibbons, 2004). High population density and reduced genetic variation in the introduced range, combined with relaxed immune functions as a consequence of initial release from pathogens, might also ultimately lead to higher susceptibility to pathogens (Lee & Klasing, 2004; White & Perkins, 2012). Several studies show either reduced or increased pathogen loads in introduced populations (e.g. Colautti et al., 2004; Schultheis et al., 2015), and similarly show mixed data in support of or against increased immune function in invasive species (Cornet et al., 2016). The eco-immunology of invasive species therefore appears dynamic and complex (Colautti et al., 2004).

Ants are among the most destructive invasive species, causing significant harm to both the environment and human economies (Holway et al., 2002). The Argentine ant (*Linepithema humile*) has been introduced from the Paraná River Basin in South America into Mediterranean and subtropical environments around the globe (Van Wilgenburg et al., 2010; Vogel et al., 2010; Wild, 2004), where it has become invasive. Its introduction pathway is well documented: from Argentina, it was introduced into Europe and the USA by the beginning of the 20th century, and from Europe invaded Australia and New Zealand, in the 1930s and the 1990s, respectively (Figure 1; Corin et al., 2007; Suhr et al., 2011; Wetterer et al., 2009; Wild, 2004). It is a problematic pest associated with a wide range of ecological and economic costs (Angulo et al., 2022; Bradshaw et al., 2016; Holway et al., 2002). In a previous study with limited sample size, we suggested that viral diversity may be slightly lower in the Argentine ant's introduced range compared to its native range (Felden et al., 2019). Similarly, the red imported fire ant (*Solenopsis invicta*) appears to have experienced a release from natural enemies in its introduced range (Porter et al., 1997; Yang et al., 2010). It has also been proposed that invasive species, including ants, could exhibit increased tolerance against pathogens and facilitate pathogen spillover into native communities, indirectly promoting their own ecological success (Cremer, 2019; Vilcinskas, 2019).

Argentine ants harbour a diversity of viruses, although their impacts on colony fitness remain unclear (Baty et al., 2020; Felden et al., 2019; Gruber et al., 2017; Lester et al., 2017; Sébastien et al., 2015; Viljakainen, Holmberg, et al., 2018). Viral infections can have detrimental effects on other ant species (*Solenopsis invicta*: Manfredini et al., 2016; Porter et al., 2013; *Lasius niger*: Schläppi et al., 2020), and some viruses are associated with stronger immune responses than others in Argentine ants (Lester et al., 2019). At the regional level in their introduced range, Argentine ants exhibit lower genetic variation compared to the native range (Suarez et al., 1999), which along with forming extensive interconnected populations (i.e. 'supercolonies') might make introduced populations more susceptible to pathogens (Tragust et al., 2015; Ugelvig & Cremer, 2012). Interestingly, population collapse has been observed in some introduced Argentine ant populations, and pathogens have been suggested as a possible contributing factor (Cooling et al., 2012; Lester & Gruber, 2016). Alternatively, the typically supercolonial social structure of invasive ants may make them particularly resilient to diseases and act as a reservoir for pathogens, disproportionately affecting native communities (Cremer, 2019).

In this study, we investigated whether introduced Argentine ant populations exhibit changes in viral loads compared to native populations, and exhibit altered immune gene expression as a result. In accordance with the theory proposed by Cremer (2019), we hypothesised that introduced Argentine ant populations are more tolerant to pathogens than their native counterparts, bearing a larger range of viruses in higher loads. In a previous study (Felden et al., 2019), we used RNA sequencing to begin exploring

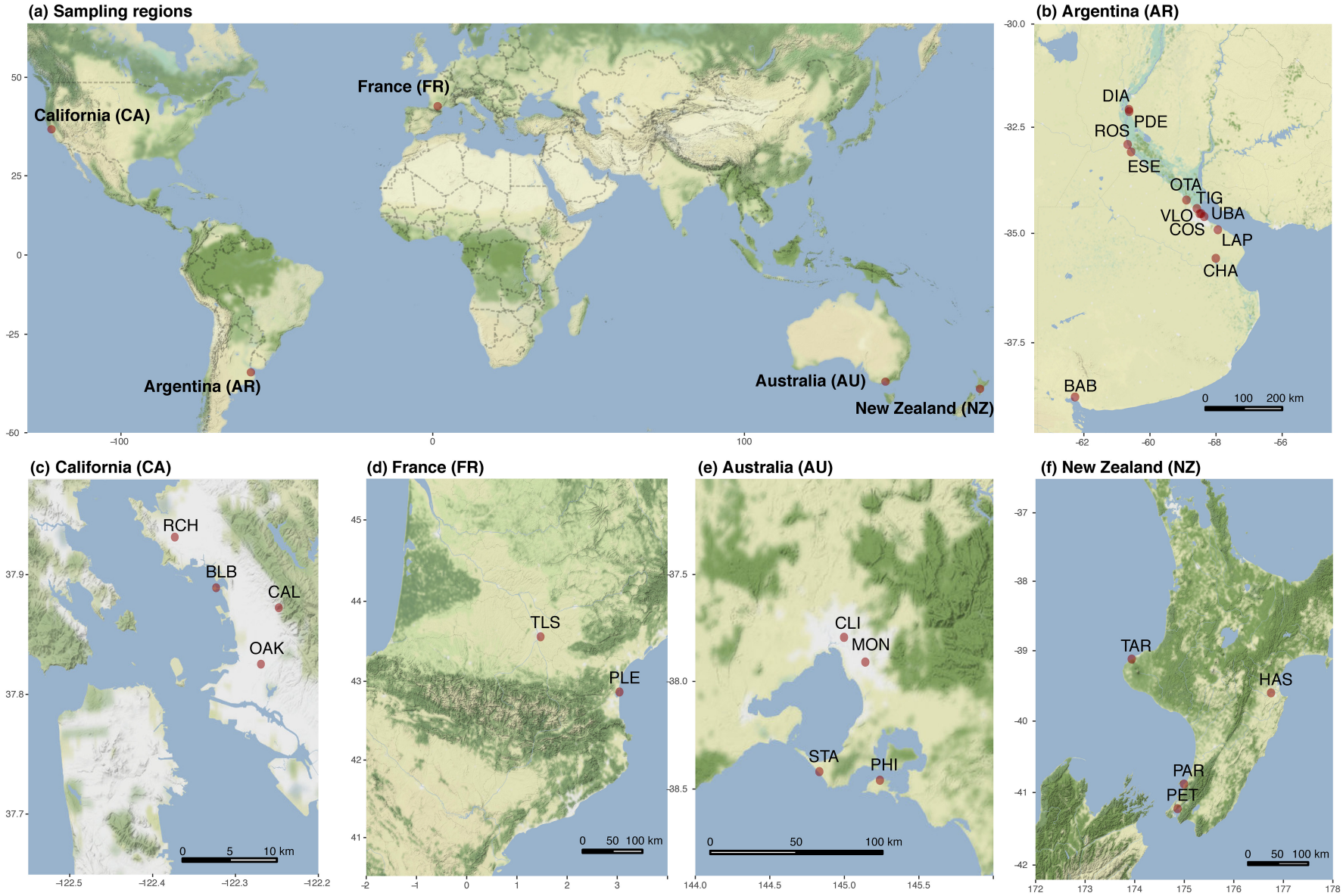

FIGURE 1 Sampling design. (a) Global distribution of sampling regions and detail of sampling locations in (b) Argentina (AR), (c) California (CA), (d) France (FR), (e) Australia (AU) and (f) New Zealand (NZ). The likely introduction pathway (Corin et al., 2007; Suhr et al., 2011; Wild, 2004) is shown with grey arrows in (a). Years of earliest record in each region are taken from Wetterer et al. (2009). Maps were drawn using the *ggmap R* package from Kahle & Wickham, (2013). Site names and descriptions can be found in Table S1.

viral diversity and transcriptome-wide gene expression along the Argentine ant introduction pathway. However, limited sample size prevented investigating correlation between immune response and individual viral loads. Using quantitative PCR (RT-qPCR) with increased sample size, we analysed viral loads for 15 viruses known to infect Argentine ants, as well as expression levels of 24 genes related to immunity and metabolism in the ant's native range in Argentina, and from four regions where it is introduced in California, France, Australia and New Zealand. We used this dataset to investigate associations among biological invasion, microbial loads and immune gene expression, and to identify which potential pathogens were present at the highest levels and were correlated with changes in immune gene expression.

2 | METHODS

2.1 | Sampling

We studied Argentine ant populations in their native range in Argentina, and in four introduced regions: USA (California), Australia (Victoria), France and New Zealand (Figure 1). Our experiment was designed to compare viral loads and immune gene expression between the ant's native range and its introduced range. In each region, large colony fragments containing queens, brood and workers were collected in summer from four sites, from 5 to 300 km apart, except in France where we collected ants from only two sites (Figure 1, Table S1). In Argentina, the populations near Rosario and Buenos Aires were selected as they are a likely source for introduced populations (Tsutsui et al., 2001). Ants from collection sites in introduced regions were part of the same supercolony. Argentine ant foraging distance has been estimated at less than 36 m (Hogg et al., 2018), therefore we considered our sites within regions distant enough to be independent. In Argentina, each site harboured a distinct colony. Upon arrival at the laboratory after field collection, colony fragments were split and maintained in standardised experimental colonies comprising 1200 workers, four queens and a small amount of brood for the purpose of a previous experiment (Felden et al., 2018, 2019). Experimental colonies were fed daily for 20 days with one mealworm and 1 mL of 20% sucrose solution and kept under a 10/14 h dark/light cycle and an average temperature of 23.5 ± 0.4°C and 58 ± 9% humidity over the four regions. Ants were therefore maintained in standardised conditions prior to sampling for molecular analyses.

Worker ants were sampled from experimental colonies in RNALater, kept at 6°C for 24 h, and at −20°C in the region of origin until shipped to New Zealand where they were stored at −80°C until RNA was extracted. Samples were made up of 20 pooled workers. The samples collected in RNALater were used for both quantitative analyses (viral loads and gene expression levels) as well as qualitative analyses (presence/absence of viruses). Foraging workers were also collected in 70% ethanol at eight additional sites in Argentina for qualitative analyses only (Figure 1, Table S1). Final sample sizes of pooled workers were $n = 24$ in Argentina (4 samples × 4 sites + 8 additional samples from 8 distinct sites for qualitative analyses only), $n = 16$ in California, Australia and New Zealand (4 samples × 4 sites) and $n = 8$ in France (4 samples × 2 sites; Table S1).

2.2 | RNA extraction and gene expression assays

Samples were homogenised in TRI reagent (Sigma-Aldrich, USA) with a Precellys Evolution homogeniser (Bertin, France) using 0.5 mm steel beads with three cycles of 20 s at 6800 rpm. We extracted RNA from 20 pooled workers using the Direct-Zol Microprep purification kit including the in-column DNase treatment with a final elution volume of 15 µL RNAse-free water (Zymo Research, USA). RNA quantity and extraction quality were checked using a Nano Photometer® (NP80, Implen, Germany). We reverse-transcribed 500 ng of RNA into cDNA using the qScript reverse transcriptase (Quantabio, USA).

The resulting cDNA was diluted with nuclease-free water to concentrations of 2.5 ng.µL^{-1}. For each sample, we added 55 µL of TaqMan® Gene Expression Master Mix to 55 µL of diluted cDNA and loaded the mixture into a 384-well TaqMan® Array Micro Fluidic Cards (Applied Biosystems, USA). The technology eliminates pipetting errors as well as potential contamination between reactions by distributing eight samples across 48 reaction wells each via centrifugation (Kikuchi et al., 2016; ThermoFisher Scientific, 2021), reducing the need for technical replication. Although reactions were run as singletons, our experimental design resulted in prioritising sample size over redundant technical replication. TaqMan® cards were processed using a QuantStudio 7 Real-Time PCR System thermocycler (Applied Biosystems) with the following thermocycling programme: (1) 50°C for 2 min; (2) 95°C for 2 min; and (3) 40 cycles at 95°C for 1 s then 60°C for 20 s. We used qPCR to target 21 immune genes, 3 metabolic genes, 15 viruses, 1 bacteriophage, 4 bacteria and 3 reference genes in the TaqMan® Array cards (Table 1). Although bacteria and phage were not the primary focus of our analysis, we included them as markers of bacterial infection. TaqMan assay primers were designed using Primer Express software (v3.0.1, Applied Biosystems/ThermoFisher Scientific, USA) except primers for black queen cell virus (BQCV), which were modified from Chantawannakul et al. (2006). For each target, we manually adjusted baseline fluorescence using the most stable values from at least four consecutive cycles across all samples prior to the threshold cycle, as well as adjusted the cycle threshold value so it sat in the exponential phase for all samples.

We measured Argentine ant gene expression and virus levels using relative quantification. We included annotated Argentine ant immune genes (Smith et al., 2011) and viruses that have been either characterised from Argentine ant samples or otherwise known to infect Argentine ants, albeit little is known about their fitness effects (Dobelmann et al., 2020; Felden et al., 2019;

TABLE 1 Grouping of genes and microbes for statistical analyses. In superscript, ND indicates targets not detected in the dataset and 1 indicates targets excluded from the overall quantitative analyses. Primer and probe sequences are given in 3′-5′ direction and amplicon product size is shown in base pairs (bp) in File S1.

Group	Gene or microbe	GenBank accession
Toll (5)	*Toll A*	LOC105680007
	Toll B	LOC105674053
	Spaetzle	LOC105678357
	Embryonic polarity protein Dorsal	LOC105677755
	Myeloid differentiation primary response protein (MyD88)	LOC105677438
RNAi (5)	*Endoribonuclease Dicer-1*	LOC105668788
	Endoribonuclease Dicer	LOC105670277
	Argonaute-2A	LOC105677214
	Argonaute-2B	LOC105669925
	Argonaute-3	LOC105679318
AMPs (2)	*Defensin-2*	LOC105675717
	Hymenoptaecin	LOC105670591
Imd/JNK (2)	*Caspase-3 (dredd)*	LOC105678455
	Fas-associated death domain protein (Fadd)	LOC105676271
JaK–STAT (3)	*Tyrosine protein kinase hopscotch*	LOC105677920
	Signal transducer and activator of transcription 5B (Stat92E)	LOC105672556
	Cytokine receptor domeless	LOC105676117
Pathogen recognition (4)	*Beta-1,3-glucan-binding protein-like A*	LOC105673881
	Beta-1,3-glucan-binding protein-like B	LOC105674418
	Peptidoglycan recognition protein LC like (PGRP-LC)	LOC105678063
	Peptidoglycan recognition protein like (PGRP-like)	LOC105672853
Metabolic genes (3)	*Cytochrome b-c1 complex subunit 2*	LOC105670362
	Fatty acid synthase like[1]	LOC105679506
	Guanylate kinase	LOC105674201
ss + RNA viruses (9)	Deformed wing virus (DWV)[1]	NC_004830
	Kashmir bee virus (KBV)	NC_004807
	Black queen cell virus (BQCV)[1]	MF417634
	Acute bee paralysis virus (ABPV)[ND]	NC_002548
	Linepithema humile virus 1 (LHUV-1)	NC_003784
	Linepithema humile C virus 1 (LhuCV1)	MH213244
	Linepithema humile bunya-like virus 1 (LhuBLV1)	MH213237
	Linepithema humile polycipivirus 1 (LhuPcV1)	MH213247
	Linepithema humile polycipivirus 1 (LhuPcV2)	MH213248
Other viruses (7)	Linepithema humile partiti-like virus 1 (LhuPLV1)	MH213239
	Linepithema humile entomopox virus 1 (LhuEV1)	MH213250
	Linepithema humile picorna-like virus 1 (LhuPiLV1)	MH213235
	Linepithema humile qinvirus-like virus 1 (LhuQLV1)	MH213241
	Linepithema humile rhabdo-like virus 1 (LhuRLV1)	MH213246.1
	Linepithema humile toti-like virus 1 (LhuTLV1)	MH213243
	Pseudomonas phage[ND]	26794211
Bacteria (4)	*Pseudomonas* spp.	MH997630.1
	Lactobacillus florum	AB498046.1
	Serratia proteamaculans	NR_025341.1
	Acinetobacter spp.	AJ303011.1

Lester et al., 2019; Viljakainen, Holmberg, et al., 2018). We used two Argentine ant genes as reference genes, that is, *Dynactin* and *PPI* (File S1). Using these two genes was established as the most stable combination of reference genes of four possible targets (i.e. *Dynactin*, *PPI*, *FIP-1* and the 16S internal reference) using NormFinder 0.953 (Andersen et al., 2004). All analyses were run in *R* 4.0.5 (R Core Team, 2021). For each sample, the mean C_t of the two reference genes was subtracted from the target gene C_t to calculate ΔC_t values. The $2^{-\Delta C_t}$ values were used for statistical analysis. In some cases, the qPCR amplification curves for *fatty acid synthase* gene, *Lactobacillus* and Linepithema humile Bunya-like virus 1 (LhuBLV1) were not suitable for accurate quantification and these targets were therefore discarded from the quantitative analyses. However, despite not being able to quantify LhuBLV1, we were able to determine presence/absence and therefore included LhuBLV1 in qualitative viral analyses. Similarly, ethanol preservation is suitable for virus detection using qPCR (Krafft et al., 2005), but samples collected in ethanol were only used in qualitative analyses as accurate quantification cannot be achieved without reference genes.

2.3 | Viral diversity in the native and introduced ranges

Our aim was to use our qPCR data in a qualitative fashion to investigate variation in viral diversity between the ant's native and introduced ranges. We analysed viral diversity using species accumulation curves in the *BiodiversityR R* package (Kindt & Coe, 2005). A virus was considered present in the sample when it was detected in the TaqMan array assay below a C_t of 35. In cases where amplification occurred but did not allow for accurate quantification, the samples were still considered positive but discarded from quantitative analyses. Prevalence for each virus was calculated as the total number of positive samples within region and compared among regions using pairwise Fisher's exact tests, reporting Bonferroni-adjusted *p*-values. We ran a PERMANOVA using the *adonis()* function in the *R* package *vegan* (Oksanen et al., 2022) computing both Bray–Curtis distance (i.e. quantitative data) and Jaccard distance (i.e. presence/absence), using 9999 permutations to test for differences in viral load composition between regions. We subsequently ran post hoc pairwise comparisons on the Jaccard PERMANOVA.

2.4 | Variation in microbial loads and gene expression in the native and introduced ranges

Our aim was to test the hypothesis of increased viral load in introduced Argentine ant populations compared to the native range, as well as variations in immune gene expression. First, we used principal component analyses (PCAs) on unscaled $2^{-\Delta C_t}$ values (i.e. on covariances and not correlations) to identify the main viruses in each region. Second, to test for differences in loads of the main viruses and immune gene expression across regions, we used mixed-effects linear modelling with Box–Cox transformed $2^{-\Delta C_t}$ values. Region was specified as the fixed effect and site as a random effect. We constructed eight multivariate linear models with response variables grouped based on prior knowledge of gene function or microbe (i.e. metabolic genes, Jak/STAT signalling, RNAi, Toll signalling, Imd/JNK signalling, AMPs, pathogen recognition genes and main viruses; Table 1). Any significant effects in multivariate analysis of variance (MANOVA) on the multivariate linear models justified further investigation of mixed-effects univariate linear models. For each model, we report significant differences in expression levels or microbial load estimates in each introduced region compared to Argentina. A significant estimate for Argentina (i.e. intercept) indicates a significant difference from 0.

2.5 | Associations between viral loads and gene expression

Our aim was to characterise the association between the key viruses we identified via PCA and immune genes. We determined associations between viral loads and gene expression using linear regressions of the first two PCs with immune gene expression within countries. Note that the original variables in the PCAs (i.e. viral loads) are transformed into new variables (i.e. PCs) through linear combinations optimised to capture the maximum amount of variation, resulting in arbitrary PC coefficient signs. We report not only Holm-adjusted *p-values* (post hoc adjustments for multiple comparisons) in the main text but also significance levels for unadjusted *p*-values as an exploratory analysis. Note that because PC coefficient signs are arbitrary, we focussed our analysis on absolute correlations.

3 | RESULTS

3.1 | Viral diversity and prevalence

We found a total of 14 viruses present in our Argentine ant samples (Figure 2, Table S2). Viral diversity in Argentina was slightly higher than in any studied region in the introduced range, but the majority of the viruses were detected in all regions, that is, Kashmir bee virus (KBV), LhuBLV1, Linepithema humile C virus 1 (LhuCV1), Linepithema humile entomopox virus (LhuEV1), Linepithema humile polycipivirus-like 2 (LhuPcV2), Linepithema humile picorna-like virus 1 (LhuPiLV1), Linepithema humile partiti-like virus 1 (LhuPLV1), Linepithema humile rhabdo-like virus 1 (LhuRLV1) and Linepithema humile toti-like virus (LhuTLV1) (Figure 2, Table S2). Other viruses were widely distributed but not ubiquitous: Linepithema humile virus 1 (LHUV-1) was found in all sampled regions except California; Linepithema humile polycipivirus-like 1 (LhuPcV1) was only found in Argentina, Australia and France;

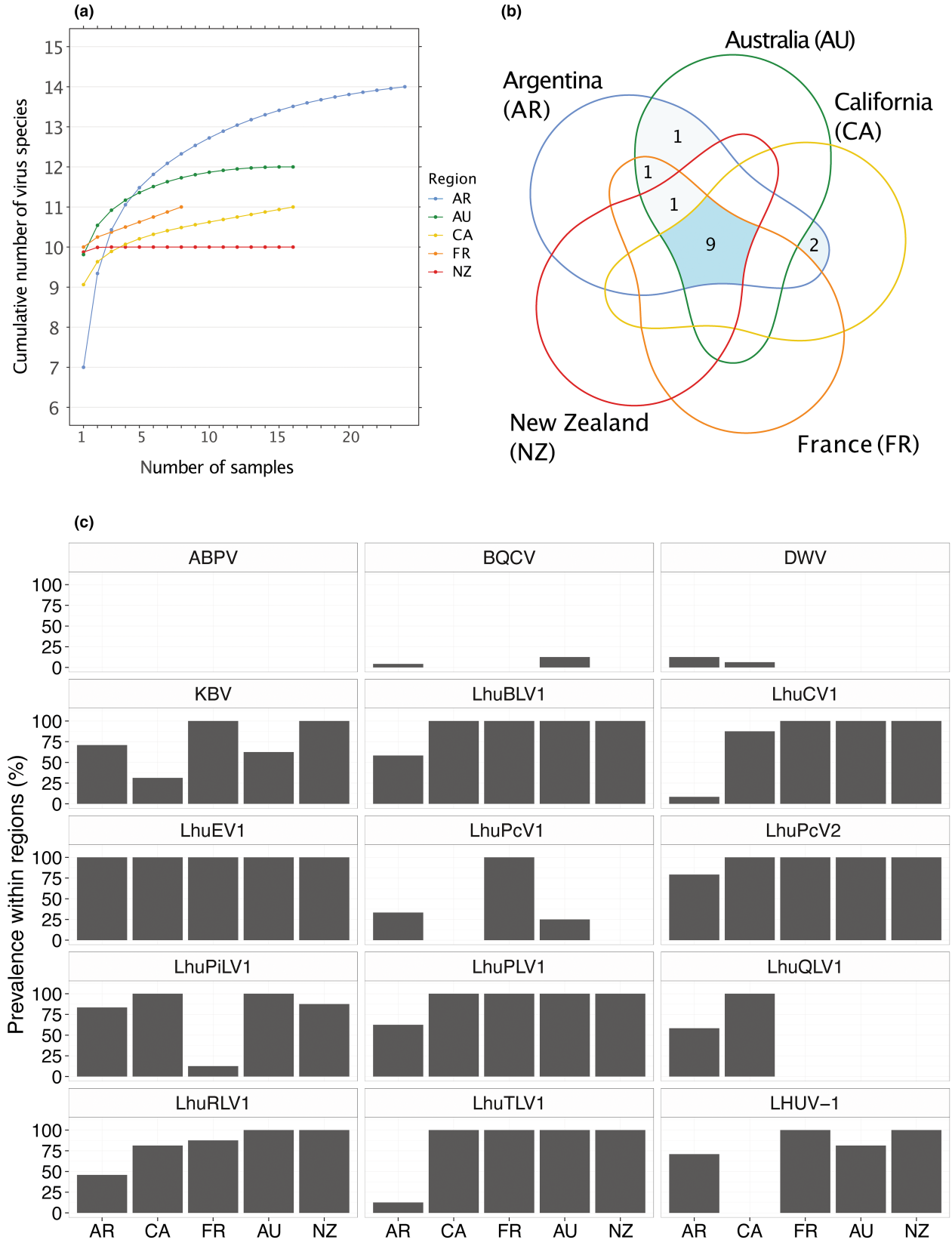

FIGURE 2 Virus diversity and prevalence. (a) Accumulation curves show lower viral diversity for selected viruses in the introduced range (CA, California; FR, France; AU, Australia; NZ, New Zealand) compared to the native range (AR: Argentina). (b) Venn diagram showing the number of viruses detected in each region. All viruses detected in our study were found in Argentina, but some viruses were absent in a subset of introduced regions. (c) Virus prevalence in samples within regions, with regions ordered following the Argentine ant's introduction pathway. Sample sizes were $n = 24$ in Argentina, $n = 16$ in California, Australia and New Zealand and $n = 8$ in France.

and Linepithema humile quinvirus-like virus 1 (LhuQLV1) was only found in Argentina and California. Deformed wing virus (DWV) was found in Argentina, California and New Zealand, and black queen cell virus (BQCV) only in Australia. Aphid bee paralysis virus (ABPV) and *Pseudomonas* phage were not detected in any of the samples.

Virus prevalence in samples within regions exhibited different patterns in relation to the introduction pathway (Figure 2, Tables S3 and S4). The prevalence of LhuCV1, LhuTLV1 and LhuRLV1 increased along the introduction pathway, particularly in the introduced range compared to Argentina (LhuCV1 and LhuTLV1: $p < .001$ for Argentina compared to all four introduced regions; LhuRLV1: $p = .003$ in Argentina compared to Australia and New Zealand but $p = .468$ and $p = .527$ in Argentina compared to California and France, respectively; Figure 2, Tables S3 and S4). There were no significant differences in prevalence among introduced regions for LhuCV1, LhuTLV1 and LhuRLV1 ($p = 1$ in all pairwise comparisons; Figure 2, Tables S3 and S4). Prevalence for LhuQLV1 was in contrast higher in Argentina and California compared to regions downstream of the introduction pathway ($p < .05$ in all cases, including Argentina vs. California; Figure 2, Tables S3 and S4). The prevalence of LhuPCV1 was high only in France, while that of LhuPiLV1 was high in every other region but France ($p < .05$ in all pairwise comparisons with France; Figure 2, Tables S3 and S4). The prevalence of LHUV-1 was high in every region except California ($p < .05$ in all pairwise comparisons with California; Figure 2, Tables S3 and S4). Prevalence of other viruses was either consistently high (LhuEV1, LhuPCV2, LhuBLV1 and LhuPLV1) or consistently low/absent (ABPV, BQCV and DWV) (Tables S3 and S4, Figure 2). The overall viral load composition analysis using PERMANOVA showed significantly distinct presence/absence patterns among regions (Jaccard distance: $p < .001$) as well as significant differences using quantitative data (Bray–Curtis distance using viral loads expressed as $2^{-\Delta Ct}$: $p < .001$; Table S5). Pairwise post-hoc comparisons on the Jaccard PERMANOVA indicated that virus presence/absence patterns were significantly different from one another in all regions ($p < .05$), except in California versus New Zealand ($p = .054$; Table S5). Non-metric multidimensional scaling analysis showed overlapping but clustered points with respect to region (Figure S1).

Using region-wise PCAs on microbial loads, we showed that the majority of variation in the viral load composition can be attributed to only a few viruses. In each region, the first two principal components (PCs) represented more than 98% of the total variance and were in all cases associated with two viruses. Overall, five viruses were identified as 'main viruses' across the five study regions. In Argentina, LhuPcV1 and LhuPiLV1 were associated with the first two principal components and accounted for 98.8% of the total variance. In Australia and California, LhuCV1 and LhuPiLV1 accounted for over 99.9% of the total variance. In France, LhuPcV1 and KBV accounted for 99.9% of the total variance and in New Zealand, LhuCV1 and LHUV-1 accounted for 99.1% of the total variance. We focused the rest of the quantitative analyses on these five main viruses. The remaining nine viruses detected were present only at very low levels (Figure 3). Furthermore, *Serratia* and *Pseudomonas* similarly did not contribute significantly to total variance (Table S10) and were therefore not included in the rest of the analysis.

3.2 | Viral loads

We found highly variable viral loads among samples (Figure 3). There was a significant effect of range on the loads of the five main viruses we identified using PCA (MANOVA: 0.221, df = 1, $F = 3.737$, $p = .005$; Table S6). However, univariate analysis could not detect differences between ranges (Table S6). Conversely, multivariate analysis at the regional level showed an effect of region on the loads of the main viruses identified via PCA, which was also detected by univariate modelling (MANOVA: 1.490, $df = 4$, $F = 7.838$, $p < .001$; Table S7). The viruses LhuPiLV1 and LhuCV1 exhibited higher loads in Australia, as did KBV in France ($p = .024$, $p = .001$, $p < .001$, respectively; Table S7).

3.3 | Gene expression

Overall, there was an effect of range on gene expression in all the immune gene categories we examined except for AMPs (MANOVAs: JaK–STAT: $p = .002$; Toll: $p < .001$; Imd/JNK: $p < .001$; AMPs: $p = .062$; pathogen recognition: $p < .001$; RNAi: $p < .001$; Table S8). Similarly, MANOVAs at the regional level show an effect of region on gene expression (MANOVAs for all immune gene categories: $p < .001$; Table S9). Pathogen recognition genes were always upregulated in the introduced range compared to Argentina (*beta-1,3-glucan-binding protein-like B*: $p < .01$ in Australia, France and New Zealand and $p < .05$ in California; *PGRP-like*: $p < .05$ in California, France and New Zealand; *PGRP-LC*: $p < .05$ in California and Australia; Figure 4, Table S9). Genes in the Toll pathway exhibited a similar trend, except *Dorsal*, which was significantly downregulated in Australia (*Myd88*: $p < .05$ in California and Australia; *Spaetzle*: $p < .05$ in Australia; *Toll A*: $p < .05$ in New Zealand; Figure 4, Table S9). Genes in the Imd/JNK pathway were also upregulated in the introduced range (*Dredd*: $p < .001$ in California, Australia and France and $p = .010$ in New Zealand; *Fadd*: $p < .001$ in France; Figure 4, Table S9). The AMP gene *Hymenoptaecin* was upregulated in France but downregulated in New Zealand compared to Argentina (*Hymenoptaecin*: $p = .002$ in France and $p = .027$ in New Zealand; Figure 4, Table S9). There was no difference in the antiviral pathway RNAi gene expression except for *Argonaut-2 B*, which was downregulated in France and Australia (*Argonaut-2 B*: $p < .01$ in both regions; Figure 4, Table S9). The JaK/STAT genes exhibited contrasting trends in California and Australia, with *Domeless* upregulated in California and *STAT92* downregulated in Australia (*Domeless*: $p < .05$ in California; *STS92E*: $p < .05$ in Australia; Figure 4, Table S9). The expression of metabolic genes was also significantly affected by range (MANOVA: $p < .001$; Table S8) and region (MANOVA: $p < .001$; Table S9). *Cytochrome b* upregulated in France ($p < .01$) and *Guanylate*

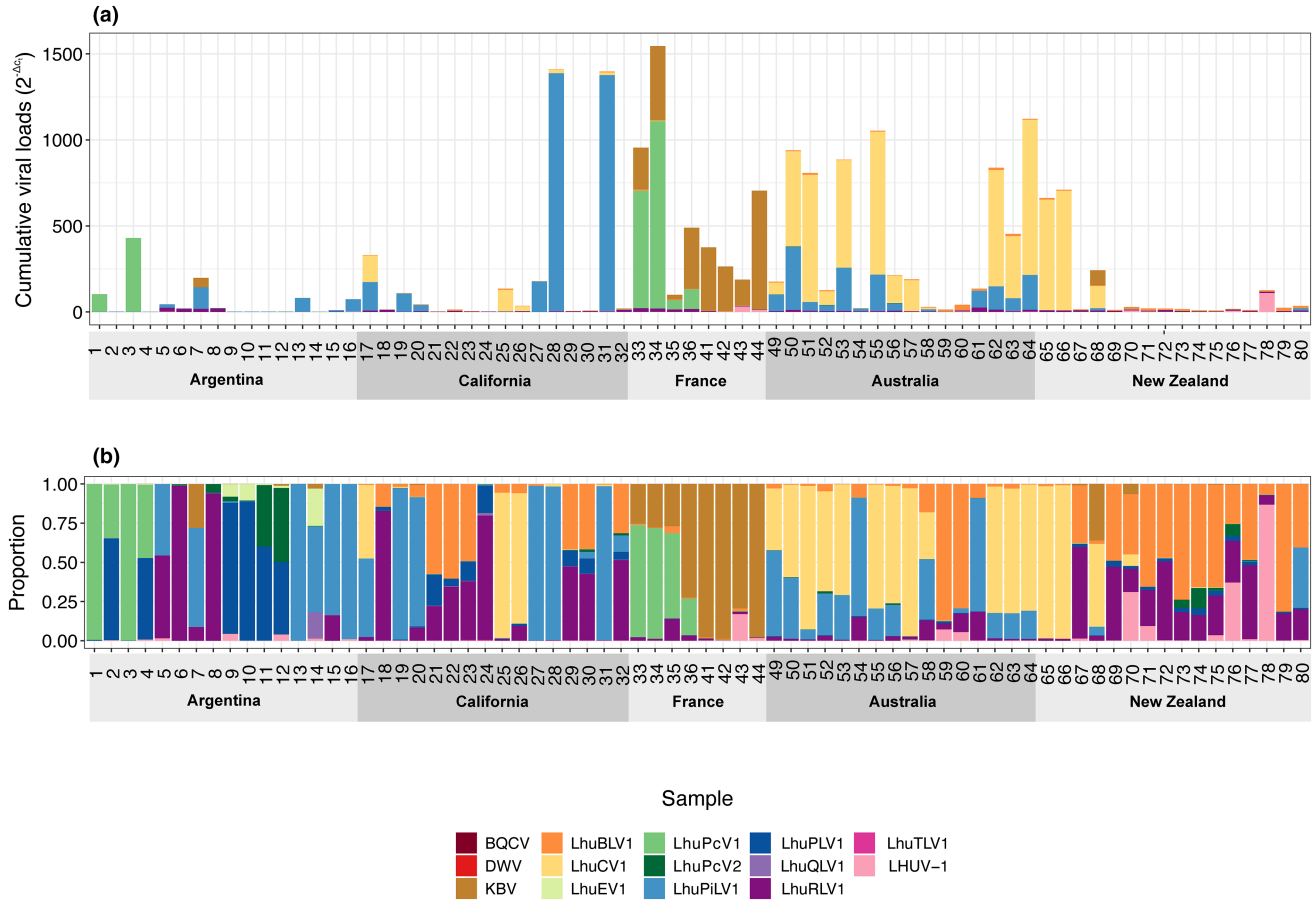

FIGURE 3 Viral loads. (a) Viral loads expressed as $2^{-\Delta C_t}$ values for selected viruses detected in Argentine ants using RT-qPCR data. (b) Viral loads expressed as proportion of all the viruses measured. Numbers on the *x*-axes indicate unique sample identification, with regions ordered following the Argentine ant's introduction pathway.

kinase downregulated in France and Australia ($p < .01$ in both regions; Figure 4, Table S9).

3.4 | Associations between viruses and gene expression

Principal component analyses of microbial loads within regions revealed that five viruses explained at least 98.8% of the total variance in viral loads: LhuPcV1, LhuPiLV1, LhuCV1, LHUV-1 and KBV (Table S10). The linear regressions between the first two principal components within regions revealed strong associations, although only 10/91 were statistically significant after Holm–Bonferroni multiple-comparison corrections. In Argentina, PC1 (associated with LhuPcV1 loads) was associated with *Toll A* expression ($\rho = .856$, $p < .001$) as well as Guanylate kinase ($\rho = .799$, $p = .018$) and PC2 (associated with LhuPiLV1 loads) was associated with *Dicer-1* expression ($\rho = .847$, $p = .002$; Figure S2). In California, PC1 (associated with LhuPiLV1 loads) was associated with *Guanylate kinase* ($\rho = -.818$, $p = .033$) and PC2 (associated with LhuCV1 loads) was associated with *Argonaut 3* ($\rho = .868$, $p < .001$; Figure S2). In France, no significant correlation was observed after multiple-comparison adjustments. In Australia, PC1 (associated with LhuCV1) was associated with *Dicer* ($\rho = -.832$, $p = .015$) as well as *Dorsal* ($\rho = -.809$, $p = .049$) and PC2 (associated with LhuPiLV1) was associated with *Dicer-1* ($\rho = .912$, $p < .001$; Figure S2). In New Zealand, PC1 (associated with LhuCV1) was associated with *beta-1,3-glucan-binding protein-like B* ($\rho = .835$, $p = .012$), *PGRP-LC* ($\rho = .891$, $p < .001$) as well as *Toll B* ($\rho = -.826$, $p = .021$; Figure S2) and no significant correlation of gene expression with PC2 held after multiple-comparison adjustments. We report significance for unadjusted *p*-values (Figure 5), which should be interpreted conservatively. Generally, this exploratory analysis showed that the main viruses' loads were significantly correlated with immune gene expression, but that these associations were not necessarily consistent across regions.

4 | DISCUSSION

We investigated variations in viral loads in introduced Argentine ant populations compared to native populations, as well as associations with immunity-related gene expression. We detected and quantified 14 potentially pathogenic viruses in Argentine ants across its

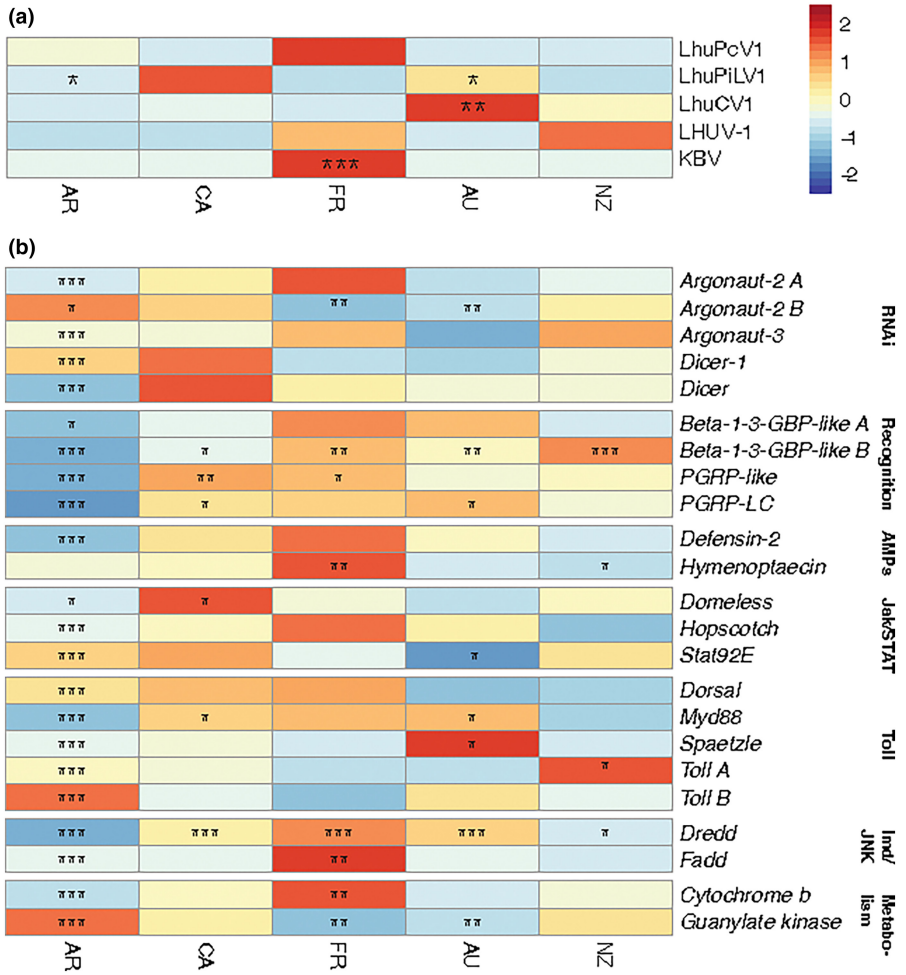

FIGURE 4 Gene expression. Heatmap shows (a) scaled viral loads and (b) scaled immune and metabolic gene expression in Argentina (AR), California (CA), France (FR), Australia (AU) and New Zealand (NZ), with regions ordered following the Argentine ant's introduction pathway. When significant, the *p*-values are indicated as * for $.01 < p < .05$, ** for $.001 < p < .01$ and *** for $p < .001$. Significance for Argentina (i.e. the intercept level) indicates model estimates different from 0.

native and introduced ranges, that is, all viruses that we surveyed except ABPV. Viral diversity appeared slightly higher in the Argentine ant's native range, supporting previous patterns observed in invasive ants (Felden et al., 2019; Yang et al., 2010). For instance, although initially characterised by an introduced population in Spain (Viljakainen, Holmberg, et al., 2018), LhuQLV1 has to date never been found downstream of the introduction pathway in Australia or New Zealand (Felden et al., 2019; Lester et al., 2019; Viljakainen, Holmberg, et al., 2018; present study). Such occurrence patterns could reflect the loss of viruses along the ant's introduction pathway. However, virus species accumulation curves did not always reach a plateau and we did not detect some of the viruses known to infect Argentine ants in some countries (e.g. KBV and DWV in New Zealand; Dobelmann et al., 2020), which indicates that our sampling design did not always fully cover the existing viral diversity in each region, or that viral infection might not be homogeneous within a region. Although most studies to date have characterised Argentine ant viruses from introduced populations (e.g. Viljakainen, Holmberg, et al., 2018; Viljakainen, Jurvansuu, et al., 2018), we did detect the majority of viruses known to infect *L. humile* in their native range, suggesting that introduced populations did not acquire novel viruses. However, the prevalence of LhuCV1, LhuTLV1 and LhuRLV1 increased sometimes dramatically in the introduced range compared to Argentina, suggesting more widespread infections in the introduced regions.

We identified a few viruses that were present in relatively high loads: LhuPcV1 (in Argentina and France), LhuPiLV1 (in Argentina, California and Australia), LhuCV1 (in California, Australia and New Zealand), KBV (in France) and LHUV-1 (in New Zealand). However, the effects of these viruses on Argentine ants, as well as their capacity to replicate within Argentine ant hosts, remain for the most part unknown (Baty et al., 2020). Interestingly, some of the viruses present in high loads in some regions were detected, but were also less prevalent in other regions. Variations in infection patterns could be due to interactions within the pathogen communities (Lester et al., 2019; Viljakainen, Holmberg, et al., 2018), host social environment (Viljakainen, Jurvansuu, et al., 2018) or wider ecosystem interactions such as proximity with other host species

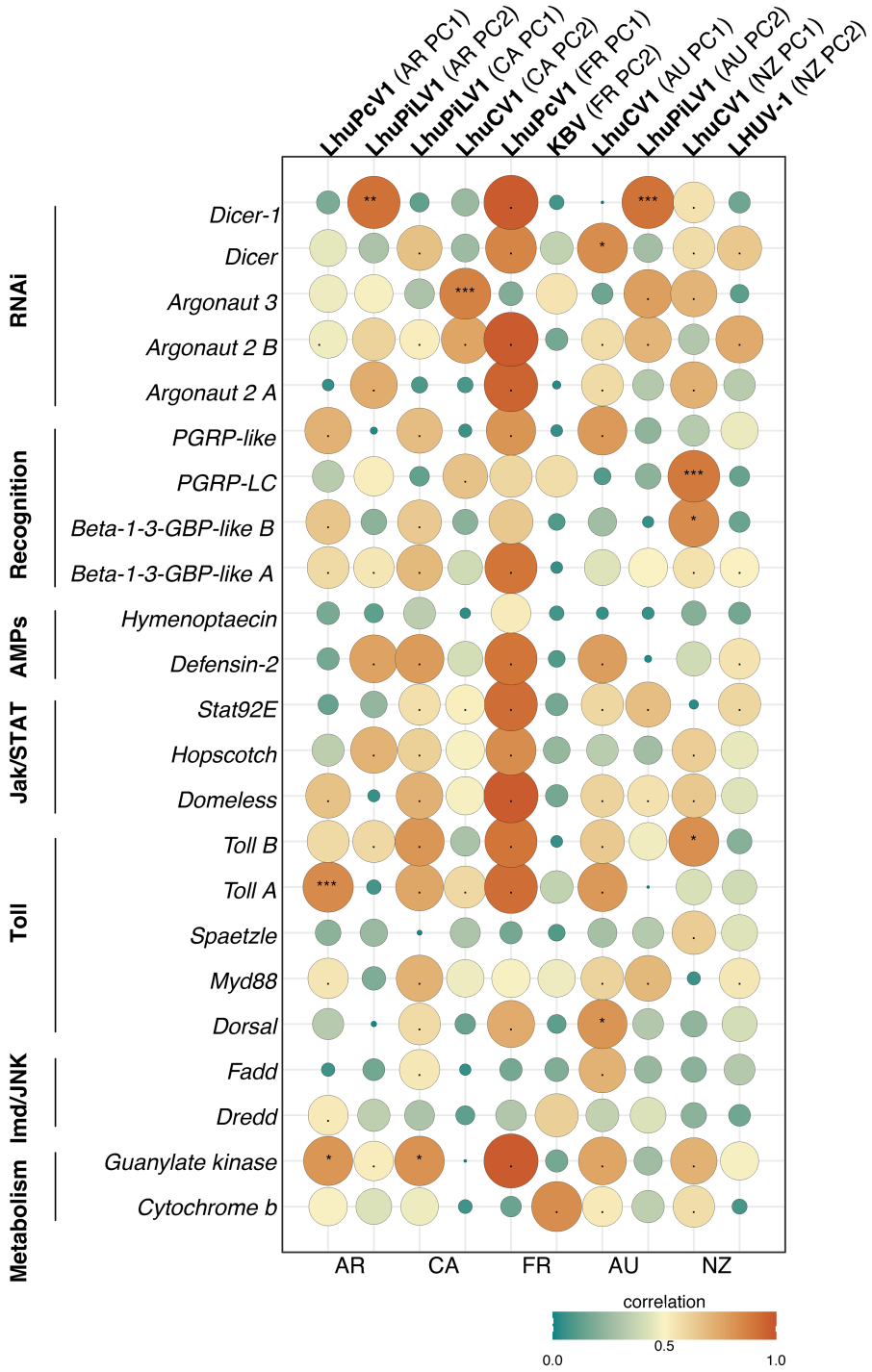

FIGURE 5 Associations between viruses and gene expression. Heatmap shows absolute values of Spearman correlations between immune genes and the first two principal components (PC) within regions (AR, Argentina; CA, California; FR, France; AU, Australia; NZ, New Zealand), with regions ordered following the Argentine ant's introduction pathway. The main viruses associated with the principal components are LhuPcV1 (AR PC1 and FR PC1), LhuPiLV1 (AR PC2, CA PC1 and AU PC2), LhuCV1 (CA PC2, NZ PC1 and AU PC1), LHUV-1 (NZ PC2) and KBV (FR PC2). Note that PC coefficient signs are arbitrary and do not necessarily reflect the direction of the relationship between immune genes and viruses associated with the PCs. When significant, the adjusted *p*-values are indicated as * for $.01 < p < .05$, ** for $.001 < p < .01$ and *** for $p < .001$. Unadjusted *p*-values <.05 are indicated with a dot.

as previously shown in Argentine ants (Dobelmann et al., 2020, 2023). Prospecting for natural enemies of invasive species has proven to be a promising tool to control the red imported fire ant, *Solenopsis invicta* (Oi et al., 2015; Valles et al., 2018), and the viruses we found in high loads might be associated with previously observed population declines (Cooling et al., 2012) and candidates to further investigate as potential biological control for the Argentine ant.

We found positive relationships between viral loads and immune gene expression, which were overall elevated in the introduced range, contrary to the expectations of the enemy release and evolution of increased competitive ability hypotheses. Viral diversity was highest in the native range, but the general trend from our analyses points to higher viral loads in the introduced range compared to the native range. Although gene expression was variable within the introduced range with significant regional differences, we found overall increased immune gene expression in the introduced range compared to the native range, especially in the earliest introduced populations in California and France. Interestingly, it has recently been shown that the strength of selection on immune genes was greater in the native range but also positively associated with time since introduction, suggesting only a short-term enemy release effect (Holmberg et al., 2024). It is likely that eco-immunological phenomena are transient in the context of biological invasions, perhaps reflected in the level of variation we observed within the introduced range. Holmberg et al. (2024) also showed that positive selection strength was the highest in RNAi-associated genes (primarily involved with defence against viruses), especially in the introduced range, suggesting the prominent role of viruses in host evolution.

The relative roles of enemy reduction and enemy release are key to understanding how pathogens may shape invasion success (Colautti et al., 2004). Our data suggest that the reduction in viral diversity associated with the introduction process that we observed did not result in a long-term release from pathogen pressure, especially in the earliest introduced populations in California and France. Rather, the high viral loads and immune gene expression we observed in the introduced range could indicate increased viral pressure compared to the native range. In the supercolonies of the invasive ant *Lasius neglectus*, *Laboulbenia formicarum* fungal infections were more prevalent than in susceptible native species (Tragust et al., 2015). In *Solenopsis invicta*, larger supercolonies also exhibited increased viral loads and diversity compared to smaller colonies (Brahma et al., 2022). Overall, we found both an increase in prevalence of some viruses, as well as generally higher viral loads in the introduced range. It is possible that despite a reduction in virus diversity, lower host genetic diversity and the formation of extensive and dense Argentine ant supercolonies could also promote pathogen transmission and select for pathogen adaptation, potentially leading to increased vulnerability to pathogens, including viral infections (Ugelvig & Cremer, 2012).

Conversely, it has been recently proposed that invasive ants may actually be able to tolerate high pathogen loads and diseases through plasticity in their social structure which can also impact native insect communities through pathogen spillover (Cremer, 2019). Social network plasticity is an effective component of social immunity (Stroeymeyt et al., 2018) and the Argentine ant does form large networks of nests, allowing for a high degree of plasticity. Furthermore, Argentine ants also carry viruses that can infect other insects (Baty et al., 2020; Dobelmann et al., 2020; Sébastien et al., 2015) and have been shown to be associated with elevated viral loads in cohabiting honey bees (Dobelmann et al., 2023). Interestingly, a recent study comparing viral communities in three sympatric ant species showed increased diversity and abundance in introduced species, including *L. humile*, which exhibited highest diversity, loads and overlap with other species (Viljakainen et al., 2023). Altogether, our results indicate an increased pathogen pressure and elevated immune gene transcription in introduced ranges compared to the native range, which suggests that introduced Argentine ant populations might indeed bear increased viral infections while still showing high ecological success.

Regional differences in virus diversity, virus infection and gene expression that we observed suggest that the effects of viral communities on Argentine ant hosts can be context dependent. We found significant correlations between the main viruses identified via PCA and certain immune and metabolic genes. However, these associations were not always consistent across regions. It is possible that region-specific pathogens that we did not investigate in our work drive the changes in gene expression that were observed, such as bacteria or fungi. In addition to variations in viral landscapes, apparent inconsistencies in our results may also be indicative of knowledge gaps in Argentine ant immune function. The Argentine ant genome annotations guiding our selection of genes for this study are derived from homologies with model organisms and better-known species such as the honey bee (Brutscher et al., 2015; Smith et al., 2011). Indeed, it is not known whether Argentine ant genes might act differently against different pathogens, pathogen combinations or even different pathogen strains, with earlier work showing complex responses to microbial communities (Abril & Jurvansuu, 2020; Lester et al., 2017; Viljakainen, Jurvansuu, et al., 2018). In addition to surveys including immune response monitoring, more functional work using pathogen inoculations or RNAi is warranted to further disentangle the roles of specific immune genes in non-model organisms (Felden et al., 2023).

Given the complexity and variability of eco-immunological parameters, surveys and correlative studies have limitations in their interpretation. For example, factors such as seasonal variation or social environment in ants can affect viral infections and immune response, respectively (Abril & Jurvansuu, 2020; Viljakainen, Jurvansuu, et al., 2018), which could explain discrepancies across study sites. Although we collected our samples in summer, it is possible that local and short-term dynamics in viral infections are confounding factors. In previous transcriptomic work using samples from the same locations as the current study, we showed expression patterns specific to immune gene categories between the native and introduced ranges (Felden et al., 2019). Toll genes were upregulated in the introduced range, but the overall trend was opposite to that of the present study for the RNAi, JaK–STAT and Imd genes with increased expression in Argentina (Felden et al., 2019). Similarly, associations between immune genes and viruses were not always consistent with a previous study (Lester et al., 2019), further indicating the importance of spatiotemporal variations in pathogen landscapes and associated immune responses.

Overall, our results pave the way to a better understanding of the eco-immunology of invasive ants. We provide data suggesting that

introduced Argentine ants might bear higher viral loads – supporting the view that large populations of invasive ants might better tolerate diseases than their native counterparts. We also outline potential primary viral pathogens found in Argentine ants and their connections to specific immune genes, which may constitute a useful basis for further functional studies using experimental inoculations and/or gene expression manipulations (e.g. using RNA interference) to better understand the effect of pathogens and their role in biological invasions.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Raw data and associated R script are available from the Dryad/Zenodo repositories at doi: 10.5061/dryad.pg4f4qrvs.

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BIOSKETCH

Antoine Felden is a molecular ecologist who studies various aspects of social insect biology. Part of his work focuses on advancing our knowledge of the eco-immunology of invasive species, from Argentine ants to the honey bee parasite *Varroa destructor*. His research aims to use an integrative approach to better understand the underlying mechanisms influencing the success of invasive species, including their parasites and pathogens.

Author contributions: A.F., M.A.M and P.J.L. designed the study. J.W.B. designed the qPCR assays. A.F., D.G.C, C.P., A.V.S. and N.D.T. participated in field work and sample collection. A.F. performed the laboratory work. J.H. and A.F. analysed the data. A.F. wrote the first draft of the manuscript, all authors substantially contributed to the peer review process and gave approval for publication.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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